THE MORTALITY OF *GALLERIA MELLONELLA* CORRELATED TO THE MULTIPLICATION OF *SERRATIA MARCESCENS* WITHIN THE GUT

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TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

Serratia marcescens Bizio is a small, gram-negative, motile, rod-shaped bacteria commonly found in water, soil, milk, bread, and other foods. It is a facultative anaerobe, grows in a wide range of pH, and is not nutritionally fastidious. The bacteria usually produce red colonies on agar but pigment production is variable and unpigmented strains are common and difficult to identify. In 1963 Bucher stated:

> A gram-negative, motile, short, rod-shaped bacterium that is isolated from a diseased insect as a likely pathogen is almost certainly *S. marcescens* regardless of its pigmentation, if it has the following characteristics: it produces little or no gas in fermentable carbohydrates; it liquifies gelatin and hydrolyzes casein; it does not deaminate phenylalanine; it does not produce arginine dihydrolase, cytochrome oxidase, or much urease; it does not form any gas in inositol and glycerol.

Only one species, *marcescens*, exists in the genus *Serratia* (Colwell, 1965). *Serratia* is variably pathogenic to insects, and in the past, many bacteria that were formerly isolated from diseased insects and named as new species were probably achromogenic strains of *Serratia*. The bacteria have shown variable pathogenicity to insects. Raun and Brooks (1963) isolated bacteria of several genera including *Aerobacter*, *Streptococcus*, *Achromobacter*, *Pseudomonas* and *Serratia* from dead larvae of *Ostrinia nubilalis*, *Agrotis ipsilon* and *Heliothis zea* collected on corn in Iowa. Infectivity tests indicated that these bacteria probably aided in the natural reduction of these insects in the field.

Bucher (1967) reported that the bacteria Serratia was the predominant pathogen in the majority of samples of hornworm larvae (Manduca sexta and M. quinquemaculata) from tobacco growing areas near Ontario, Canada. However, the bacteria caused little harm unless the insects were also exposed to certain stress factors. Bracken and Bucher (1966) found that the bacteria Serratia marcescens was present in laboratory cultures of wax moth larvae and caused a fatal septicemia in emerged adults of an ichneumonid, Exeristes comstockii, that had been reared on the larvae.

In the course of experiments designed to study the principles of infections and immunity in insects Metalnikov (1930) found the larvae of the wax moth Galleria mellonella to be highly susceptible to Serratia when the latter was injected into the hemocoel but quite resistant to infection per os. He explains the difference on the assumption that Galleria possesses a naturally acquired immunity because it lives on food open to contamination. The bacteria multiplies extracellularly in the blood and body cavity of Galleria and produce a generalized septicemia that kills the host rapidly, usually within 1 to 3 days. Bucher stated in 1963 that for bacteria injected directly into the hemocoel the LD_{50} was about 40 bacteria for Galleria.

Serratia has been combined with other bacteria in attempts to obtain a synergistic mortality reaction. In

1959, Stephens found no synergistic effect in combination of Pseudomonas aeruginosa with Serratia on the grasshopper, Melanoplus bivittatus. Serratia induced a constant mortality (of about 40%) in both xenic-corn-fed and axenic-diet-fed caterpillars. When P. aeruginosa and S. marcescens were applied simultaneously to axenic-diet-fed larvae, the induced mortality was about 50%, intermediate between the mortalities caused by either bacterium alone. This confirmed the results of Stephens (1959) who found that these two facultative pathogens do not act synergistically.

Comparable results have been obtained by Steinhaus (1959) on the wax moth Galleria; while Bacillus thuringiensis helped Serratia to act as a secondary invader, Serratia actually reduced the efficiency of B. thuringiensis. These observations of Steinhaus were supported by the results reported by Isakova (1963). She found that at doses not high enough to kill larvae rapidly by toxicosis due to the crystal toxin, larvae died more slowly due to septicemia. The mortality induced by a mixture of B. thuringiensis with gram-negative bacteria like Pseudomonas, Serratia, Enterobacter, and Flavobacterium was never higher than that caused by the most effective component, B. thuringiensis (Burges and Hussey, 1971). Krieg (1970) found that antagonism between B. thuringiensis strains is caused by bacteriocins. Bacteriocins of another type are well-known from some gram-negative potential pathogens such as . . . Serratia marcescens (Prinsloo, 1966).

Weakening of insect host by toxins may favor bacterial infection. For example, Lepesme (1938) obtained no mycosis after application of Aspergillus flavus to locusts but obtained instead a secondary infection by Serratia. However, the bacteria have no part in the mortality but are a secondary invader. Artificial infection with bacteria may have a greater effect in populations having latent virus infections than in healthy ones. For example, food treated with Serratia induced an outbreak of nuclear polyhedrosis causing 100% mortality in a latently infection population of Neodeprion sertifer.

Against a background of variable pathogenicity, Serratia infections have often been considered diseases primarily found in laboratory insect cultures. According to Bucher, there are no records of it causing an epizootic in a field population (1963). Steinhaus (1959) found no data indicating spontaneous infection among insects in nature. Serratia is more commonly isolated from insects under laboratory cultivation than in insects taken from the field. Many records of its pathogenicity are based only on information from isolated insects without confirmatory experimental tests. The laboratory nature of the pathogenicity of Serratia indicates that certain stress factors occur under laboratory conditions that do not occur in nature.

In 1959, Steinhaus classified Serratia as a facultative pathogen, indicating that Serratia had shown some

mechanism that caused damage to susceptible body or gut tissue. At that particular time, Serratia was classified as a facultative pathogen because it was "likely to possess some positive ability to invade the hemocoel."

If Serratia is to be classified as a facultative pathogen, the reasoning for doing so should be clarified. The intent of this study was to make more clear the role Serratia plays as a pathogen of Galleria. Serratia causes variable mortality of Galleria by per oral microinjection (Steinhaus, 1959). Experimentation has shown that some Serratia strains produce lecithinase (Esselmann and Liu, 1961) and toxins (Liu, 1961). The purpose of this investigation was to test the hypothesis that the ability of Serratia to kill Galleria is directly correlated with the ability of Serratia to multiply in the gut.

METHODS AND MATERIALS

The strains of Serratia used in this study were obtained from the lyophilized culture collection of the Division of Insect Pathology at the University of California, Berkeley. In selecting the strains it was assumed there was enough variation among the strains to indicate variable pathogenicity with testing.

Preliminary testing was performed on 13 strains of S. marcescens which were isolated from various insects, fruit, or possibly humans. Full strength 16 hour peptone water

(1% bacto-peptone + .5% NaCl) cultures of Serratia were used. The test insects (Galleria mellonella) were reared at 30°C on a medium consisting of 1200 cc Pablum mixed cereal, 100 ml water, 100 ml glycerin, and 100 ml honey. Larvae were stored at 10°C until needed and the last or the next to last instar larvae were used. Approximately 1000 larvae were used for the microinjection experiments. In the forced feeding experiments (per os), .003 ml of inoculum was introduced into the Galleria alimentary tract (per os) through a self-fabricated glass-tipped 27 gauge needle with syringe mounted on a microinjector. Next, after washing the ventral surface of Galleria larvae with a 1% solution of Hyamine 10X, inocula were injected intrahemocoelically through one of the first pair of prolegs by a 27 gauge needle with the same 1 ml B-D Yale Tuberculin syringe calibrated with microinjector to deliver 0.003 ml of inoculum per injection. For the initial full strength experiments, five Galleria larvae were injected intrahemocoelically and per os as were also five control intrahemocoelic and five control per os larvae. The mortality was checked every 24 hours.

Two criteria were used to select the six Serratia strains to be used in the study. First, a wide range of per os mortality was chosen. This was done to distinguish differences in virulence among the strains during later experiments. Secondly, strains were also chosen on the basis of pigment production; three were pigmented and three were

nonpigmented. A possibility considered early in the study was that virulence might change with loss or gain of pigment production.

Taxonomic studies were conducted to positively identify that the strains being used were Serratia. According to Bucher (1963) the following tests identified Serratia (see Table 1).

Possible Serratia toxins were tested for virulence in the following manner. The supernatant of Serratia cultures was injected intrahemocoelically and per os into a total of 40 insects. Sixteen hour peptone water shake cultures were spun for 30 minutes on a clinical centrifuge. The supernatant was purified by millipore filtration and microinjected intrahemocoelically and per os into 20 Galleria larvae, alternating with nutrient agar + .2% yeast extract (NA + Y) plates as controls.

The 6 strains of Serratia were next tested to determine the pH where each grows best; the following range of pH was used: 5.8, 6.4, 7.0, 8.4, 10.0. Each strain was cultured for 16 to 18 hours in 100 ml peptone water shake cultures adjusted to the pH values by 40% NaOH or 1% HCl. Percentage absorbance was read on a Bausch and Lomb Spectronic 20 Spectrophotometer to determine crude approximation of bacterial growth at different pH values. Since the gut pH of Galleria is 8.4 and the pH range of the blood is 5.8-6.4 (Wigglesworth, 1950), any variation of growth between strains at differing pH may possibly relate to virulence.

Table 1. Diagnostic tests for *Serratia marcescens*

1.	Gram Stain	-
2.	Glucose fermentation	+
3.	NO_3 to NO_2 reduction	+
4.	Flagella	peritrichous close coiled
5.	T-7 + TTC	+
6.	Methyl Red	-
7.	Voges-Proskauer	+
8.	Simmons Citrate	+
9.	H_2S (TSI)	-
10.	Phenylalanine deaminase	-
11.	Sorbitol	+
12.	Raffinose	-
13.	Arabinose	-
14.	Gelatine	+
15.	Arginine dihydrolase	-
16.	Cytochrome oxidase	-
17.	Urease	-
18.	Casein hydrolysis	+
19.	Lecithinase	+

Dilution experimentation with 6 hour shake cultures of Serratia in peptone water indicated that dilutions of 1/50,000 and 1/100,000 gave approximately 0 to 10 cells and 10 to 20 cells, respectively, with each inoculation on NA + Y plates. (Earlier full strength inoculations exceeded 1 million cells.) Each resultant colony was counted as having originated from a single cell. The culture density was standardized with a Bausch and Lomb Spectronic 20 Spectrophotometer and cultures with 30 to 40% absorbance were primarily used for injections.

Galleria were injected with each strain into the hemocoel through a first proleg and per os between the mandibles. At the same time, alternating with the insect injections, an equal number of injections of each inoculum were made on NA + Y test plates for subsequent counting of colonies. Larvae were then placed in glass petri dishes on moistened filter paper with a small amount of rearing medium. Mortality was recorded every 24 hours.

Two series of the per os injected larvae were selected to see if bacterial multiplication could occur in the gut of the insect. Two larvae were sacrificed from each strain dilution per day over a period of 5 days. The oral and anal ends of the larvae were sealed with a vaseline-water mixture, and the larval exterior was sterilized in a 40% sodium hypochlorite solution. After rinsing in sterile water, the larvae were triturated using a mortar and pestle with 5 ml

sterile water. A loop of the Galleria-water mixture was diluted in a 2 ml sterile water blank. Next a loop of the dilution was streaked on a tergitol-7 + triphenyltetrazolium chloride (T-7 + TTC) test plate. The plate was incubated 24 hours at 30° and examined for characteristic Serratia colony color, colony margin, and blue color change in the background medium as were shown by plates streaked earlier with the pure strain.

DATA AND DISCUSSION

Preliminary tests involving 13 strains of Serratia, isolated and maintained in the Insect Pathology laboratories at the University of California, Berkeley, indicated that the bacteria have at least potential pathogenicity for Galleria. This was verified by 100% mortality of the insect following full strength injection of a culture into the hemocoel. However, Serratia is considerably less pathogenic when introduced per os. As indicated by Table 2, variable rates of per os mortality were shown by the 13 strains. The selection of strains 0-8-9, 0-10-1, 0-41-1, 0-34-1, 0-34-5, and 0-34-6 gave a wide variation of per os mortality. Three of these strains were pigmented and 3 non-pigmented.

Pigment production of the bacteria did not change during the course of the study and thus it was not correlated with virulence. Change in chromogenesis would have occurred more readily if transfers of the Serratia strains had been

Table 2. *Serratia* preliminary tests indicating intrahemocoelic and per os mortality with *Galleria*.

Strain #	Source	Chromo-genesis	IH Killed	Total Inj.	% Mort.	PO Killed	Total Inj.	% Mort.	Chosen?
0-8-2	Silkworm	-	5	5	100	1	5	20	
0-8-9	Locust (Greece)	+	5	5	100	0	5	00	Yes
0-9-1	Apples - Fruit	+	5	5	100	4	5	80	
0-9-2	Lab: housefly/ *Drosophila*	+	5	5	100	3	5	60	
0-9-4	Possibly human	+	5	5	100	2	5	40	
0-10-1	Tent caterpillar	+	5	5	100	5	4	80	Yes
0-12-4	Potato tuber moth	-	5	5	100	1	5	20	
0-34-1	*Bombyx mori* caterpillar	-	5	5	100	3	5	60	Yes
0-34-2	*Musca domestica*	+	5	5	100	4	5	80	
0-34-5	*Megachille*	-	5	5	100	4	5	80	Yes
0-34-6	*Saperda carcharias* L. dead larvae	-	5	5	100	1	5	20	Yes
0-36-1	Catalpa Sphinx moth	+	5	5	100	4	5	80	
0-41-1	*Chiloterea infuscatella*	+	5	5	100	5	5	100	Yes

made through broth culture to broth inoculations instead of single colony to broth inoculation.

Taxonomic studies were conducted in the study to positively identify that the strains being used were Serratia. According to Bucher (1963), the following tests positively identify S. marcescens. The results of the tests used for Serratia are indicated in tabular form (see Table 3). The lecithinase test mentioned previously as important to the interpretation of the gut multiplication test was positive for all strains, and thus no correlation could be made with variable strain pathogenicity.

In the toxin production study, test plates indicated that no cells were injected along with the supernatant. The Galleria larvae showed no response to the injections but this does not rule out production of exotoxins by the strains. It would clearly indicate, however, that more than the supernatant containing possible exotoxins from Serratia are necessary to kill Galleria by intrahemocoelic or per os injection. To improve the validity of this test, the toxins should be identified.

Serratia grew in a wide range of pH as was indicated by the results of the pH tolerance test conducted. All strains grew well at pH 5.8, 6.4, 7.0, and 8.4, but little or no growth was given at pH 10.0. Since there was no variation in strain growth at different pH, there remain possibly other factors not indicated in this study which enhance or retard the multiplication of Serratia in the gut of Galleria.

Table 3. *Serratia* diagnostic test results

Test	0-8-9	0-41-1	0-10-1	0-34-1	0-34-5	0-34-6
1. Gram stain	-	-	-	-	-	-
2. Glucose fermentation	+	+	+	+	+	+
3. NO_3 to NO_2 reduction	+	+	+	+	+	+
4. Flagella	-	+	+	+	+	+
5. T-7 + TTC	+	+	+	+	+	+
6. Methyl Red	-	-	-	-	-	-
7. Voges-Proskauer	+	+	+	+	+	+
8. Simmons Citrate	+	+	+	+	+	+
9. Phenylalanine deaminase	-	-	-	-	-	-
10. H_2S (TSI)	-	-	-	-	-	-
11. Sorbitol	+	+	+	+	+	+
12. Raffinose	+	+	+	+	+	+
13. Arabinose	+	-	-	+	+	+
14. Gelatine	+	+	+	+	+	+
15. Arginine dihydrolase	-	-	-	-	-	-
16. Cytochrome oxidase	-	-	-	-	-	-
17. Urease	-	-	-	-	-	-
18. Casein hydrolysis	+	+	+	+	+	+
19. Lecithinase	+	+	+	+	+	+

Pathogens are indeed virulent if a few of their cells can kill as effectively as millions of them. Thus, to indicate more clearly the virulence of Serratia, dilutions of full-strength peptone water cultures were made by standard microbiological techniques. Since the different strains reached the logarithmic growth phase within variable time intervals, a Bausch and Lomb Spectronic 20 Spectrophotometer reading was used to roughly indicate growth within the 6 hour peptone water shake cultures. Three trials for each strain indicated that dilutions of 1/50,000 and 1/100,000 normally produced 10-20 or 0-10 cells respectively when a reading of near 30% absorbance was previously obtained on the Bausch and Lomb Spectrophotometer.

Strains chosen in this study were able to cause mortality when injected into the hemocoel. Furthermore, Table 4 indicates that 4 of the 6 chosen strains, 0-10-1, 0-41-1, 0-34-1, and 0-34-5, were more effective pathogens, regardless of the dilution (number of cells injected). Many authors have expressed serious reservations about the practicality of intrahemocoelic injections. First, there is no method in nature for such an injection, and second, many authors speculate that the natural flora of the insect gut would probably cause the same mortality if microinjected into the hemocoel.

The six strains caused reduced per os virulence when compared to the intrahemocoelic virulence. Full strength

Table 4. Results of insect mortality and plate counts using three different dilutions of *Serratia*.

A - Full Strength, no dilution
B - Dilution of 1/50,000
C - Dilution of 1/100,000

Experiment no.*	Dilution	Bacterial Plate Counts Ave. no. cells (range)	No. Killed	Total inj.	% Killed
Strain no. 0-8-9					
1	A	(no plate count)	26	30	87
	B	1.7 (0-7)	1	10	10
	C	0.7 (0-2)	1	10	10
2	A	(no plate count)	8	30	27
	B	4.1 (0-7)	0	10	0
	C	1.5 (0-6)	0	10	0
Strain no. 0-10-1					
1	A	(no plate count)	30	30	100
	B	4.1 (1-8)	10	10	100
	C	0.0 (0)	1	10	10
2	A	(no plate count)	24	30	80
	B	5.0 (2-8)	5	10	50
	C	2.0 (0-5)	2	10	20
Strain no. 0-41-1					
1	A	(no plate count)	30	30	100
	B	14.6 (4-22)	9	10	90
	C	9.3 (5-14)	9	10	90
2	A	(no plate count)	27	30	90
	B	6.3 (1-20)	13	20	65
	C	2.1 (0-6)	3	20	15
Strain no. 0-34-1					
1	A	(no plate count)	30	30	100
	B	8.8 (2-14)	9	10	90
	C	1.7 (0-4)	7	10	70
2	A	(no plate count)	27	30	90
	B	11.0 (1-19)	14	20	70
	C	5.2 (3-9)	8	10	80

Table 4. (Continued)

Experiment no.*	Dilution	Bacterial Plate Counts Ave. no. cells (range)	No. Killed	Total inj.	% Killed
Strain no. 0-34-5					
1	A	(no plate count)	29	30	97
	B	32.0 (10-52)	9	10	90
	C	13.6 (2-21)	6	10	60
2	A	(no plate count)	22	30	73
	B	7.8 (2-15)	6	20	30
	C	3.5 (0-11)	6	20	30
Strain no. 0-34-6					
1	A	(no plate count)	26	30	87
	B	36.3 (15-47)	1	10	10
	C	15.4 (7-27)	1	10	10
2	A	(no plate count)	7	30	23
	B	14.0 (3-43)	1	20	5
	C	4.1 (0-16)	1	20	5

*Experiment no. 1 refers to intrahemocoelic injections; Experiment no. 2 refers to per os injections.

injections produced rather high mortality in four of the six strains, but pathogenicity decreased noticeably with decrease in the number of cells microinjected. However, two strains, 34-1 and 41-1, were apparently more consistent in producing high mortality using differing number of cells. Strains 0-8-9 and 0-34-6 produced low levels of mortality while 0-10-1 and 0-34-5 gave intermediate results.

Some strains of Serratia were shown to be more effective in multiplying in the gut of Galleria than were other strains. Strains 0-41-1 and 0-34-1 indicated ability to multiply in the gut greater than 50% of the time while strain 0-10-1 showed multiplication 45% of the time. Strains 0-8-9, 0-34-5, and 0-34-6 indicated a low percentage of gut multiplication. Table 5 indicates the multiplication data and data by percentage among the 20 larvae injected for each strain.

It cannot be stated that a direct correlation between pathogenicity and ability to multiply in the gut has been shown in all cases. However, it is quite possible that this is the case as shown in Table 6. Strain 0-34-1 indicated a reasonably close correlation between strong per os mortality and strong per os gut multiplication. Strain 0-8-9 produced zero per os insect mortality and low per os gut multiplication of the bacteria. Strains 0-10-1, 0-41-1, and 0-34-5 gave a close correlation between per os mortality and per os multiplication on one of the dilutions performed, but gave inconsistent results with the other.

Table 5. Percentage multiplication of *Serratia* in the gut of *Galleria*.

Dilution 1/50,000

Strain No.	Bacterial Plate Counts Ave. no. cells	Range	# Killed	Total Inj.	% Killed
0-8-9	2.6	0-10	1	20	5
0-10-1	5.5	2-11	10	20	50
0-41-1	7.1	1-23	13	20	65
0-34-1	11.1	1-34	12	19	63
0-34-5	9.5	3-28	12	18	67
0-34-6	12.6	2-49	7	20	35

Dilution 1/100,000

Strain No.	Bacterial Plate Counts Ave. No. cells	Range	# Killed	Total Inj.	% Killed
0-8-9	2.3	0-6	0	10	0
0-10-1	2.0	0-4	9	10	90
0-41-1	3.7	0-12	11	20	55
0-34-1	5.2	0-8	6	10	60
0-34-5	4.1	1-10	7	20	35
0-34-6	6.3	0-19	8	20	40

Table 6. Correlation between per os gut multiplication and per os mortality.

Strain No.	Dilution 1/50,000 Bacterial Plate Count Ave. No. Cells	Range	# Killed or Recovered	Total Inj.	% Killed or Recovered
MPO 0-8-9	2.6	0-10	1	20	5
PO 0-8-9	4.1	0-7	0	10	0
MPO 0-10-1	5.5	2-11	10	10	50
PO 0-10-1	5.0	2-8	5	10	50
MPO 0-41-1	7.1	1-23	13	20	65
PO 0-41-1	6.3	1-20	13	20	65
MPO 0-34-1	11.1	1-34	12	19	63
PO 0-34-1	11.0	1-19	14	20	70
MPO 0-34-5	9.5	3-28	12	18	67
PO 0-34-5	7.8	2-15	6	20	30
MPO 0-34-6	12.6	2-49	7	20	35
PO 0-34-6	14.0	3-43	1	20	5

Table 6. (Continued)

Dilution 1/100,000

Strain No.	Bacterial Plate Count Ave. No. Cells	Range	# Killed or Recovered	Total Inj.	% Killed or Recovered
MPO 0-8-9	2.3	0-6	0	10	0
PO 0-8-9	1.5	0-6	0	10	0
MPO 0-10-1	2.0	0-4	9	10	90
PO 0-10-1	2.0	0-5	2	10	20
MPO 0-41-1	3.7	0-12	11	20	55
PO 0-41-1	2.1	0-6	3	20	15
MPO 0-34-1	5.2	0-8	6	10	60
PO 0-34-1	5.2	3-9	8	10	80
MPO 0-34-5	4.1	1-10	7	20	35
PO 0-34-5	3.5	0-11	6	20	30
MPO 0-34-6	6.3	0-19	8	20	40
PO 0-34-6	4.1	0-16	1	20	5

MPO - per os gut multiplication.
PO - per os mortality.

Variation in the host insect was considerable. Further studies should use the same instar, and the insect larvae should be weighed. Individual insect resistance is probably too great a variable to leave at random.

CONCLUSIONS

It cannot be determined by this study why Serratia is a facultative or potential pathogen for Galleria and how the bacteria may be made to act reliably as an obligate pathogen. However, certain characteristic modes have been determined whereby Serratia can kill Galleria. Since from 0-10 cells of Serratia are pathogenic when experimentally introduced into the hemolymph of Galleria larvae, Serratia is at least characteristic of Bucher's (1960, 1963) group of potential pathogens.

The primary factor in the production of per os mortality quite possibly is the ability of Serratia to multiply in the gut. If Serratia can establish an alimentary tract infection of sizeable proportions, other factors such as lecithinase or toxin production may be sufficient to enhance the virulence of Serratia and enable it to kill the Galleria larvae.

Multiplication in the gut as a primary factor in pathogenesis should be integrated as only one of several factors that may influence pathogenicity. The gut is a tremendous churn of activity. Studies on temperature,

external humidity, food, digestive fluids and secretions, bactericidal substances, the nature of the gut wall in Galleria may strengthen the hypothesis that ability to multiply in the gut is a prime factor in the pathogenesis of Serratia. These, however, are studies relating to Galleria more than to different strains of Serratia.

Serratia is more of a problem in laboratory or insectary-reared insects than among insects in nature. The pathogenicity of Serratia marcescens in Galleria mellonella was shown in this study and it was determined that some strains of Serratia can be classified as facultative while some are only potential pathogens. The degree of pathogenicity was correlated with the ability of Serratia to multiply in the gut of Galleria.

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